PB84-201714

In Vivo/In Vitro Approach to the Toxicological Assessment of Hazardous Waste

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

Jun 84

U.S. Department of Commerce
National Technical Information Service
NTIS

PB84-201714

EPA-600/D-84-151
June 1984

An *In Vivo*/*In Vitro* Approach to the Toxicological Assessment of Hazardous Waste

by

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TECHNICAL REPORT DATA *(Please read Instructions on the reverse before completing)*		
1. REPORT NO. EPA-600/D-84-151	2.	3. RECIPIENT'S ACCESSION NO. PB84 201714
4. TITLE AND SUBTITLE An in vivo/in vitro approach to the toxicological assessment of hazardous waste		5. REPORT DATE June 1984 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Joellen Lewtas* and Barbara Andon**		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS * USEPA, Health Effects Research Laboratory Research Trianlge Park, NC 27711 and **Environmental Health Research and Testing P.O. Box 12199, RTP, NC 27709		10. PROGRAM ELEMENT NO. ABDVIA 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS USEPA, Health Effects Research Laboratory Research Trianlge Park, NC 27711		13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/11
15. SUPPLEMENTARY NOTES		

16. ABSTRACT

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The protocol, called the Tox Screen, involves the oral administration of waste material to rodents for 10 consecutive days. At the end of the 10-day period the whole animal, body tissues and fluids are evaluated for toxicity. A major goal of the Tox Screen is to maximize the amount and type of potential health effects information that can be obtained by exposing metabolically competent intact animals to complex waste mixtures.

The Tox Screen is being validated by using a series of positive (and negative) control compounds of known and defined toxicity. The validation study ensures that the protocol will be capable of detecting biologically active wastes and will allow assessment of its use as a predictive tool for known chronic effects.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
18. DISTRIBUTION STATEMENT Release to Public	19. SECURITY CLASS (This Report) Unclassified 20. SECURITY CLASS (This page) Unclassified	21. NO. OF PAGES 30 22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

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Abstract

An *in vivo*/*in vitro* toxicological screen has been developed to evaluate potentially hazardous waste samples and process stream residuals. The biological methods employed in the screen are designed to be rapid, inexpensive methods capable of screening large numbers of wastes. Emphasis is placed on identifying a wide range of potential toxic responses associated with each waste by employing diverse test methods. The toxic endpoints identified by the screen include mutagenesis/carcinogenesis, general toxicology, neurotoxicology, reproductive toxicology, teratology, and immunotoxicology.

The protocol, called the Toxicological Screen, involves the oral administration of waste material to rodents for 10 consecutive days. At the end of the 10-day period the whole animal, body tissues and fluids are evaluated for toxicity. A major goal of the Toxicological Screen is to maximize the amount and type of potential health effects information that can be obtained by exposing metabolically competent intact animals to complex waste mixtures.

The Toxicological Screen is being validated by using a series of positive (and negative) control compounds of known and defined toxicity. The validation study ensures that the protocol will be capable of detecting biologically active wastes and will allow assessment of its use as a predictive tool for known chronic effects. An additional goal of the validation study is to identify the assays which most readily detect the toxic potential of the positive control compounds. These selected assays will constitute a Prescreen for the entire protocol. The Prescreen can then be used as a rapid means of prioritizing waste samples to be evaluated in the full Toxicological Screen.

Initial trials of the validation study with four positive control compounds demonstrate the Toxicological Screen's ability to detect biologically active chemicals and to generate dose response data.

Key Words: Hazardous wastes, industrial wastes, environmental pollutants, toxic wastes, toxicity testing, hazardous waste assessment, solid waste, mutagenesis/carcinogenesis, general toxicology, immunotoxicology, neurotoxicology, reproductive toxicology, and teratology.

Introduction

The disposal of the vast quantity of wastes generated in the United States each year is of great economic, public and environmental concern. The development of suitable waste management methods requires a means of identifying the "hazardous" proportion of the nations total wasteload. It is only with the identification of hazardous wastes that proper storage, transportation and disposal methods can be utilized to ensure public health and safety and integrity to the environment.

Over the past 10 years much research has been directed toward the identification and hazard assessment of a variety of complex mixtures and environmental pollutants[1]. Many programs have relied on a totally *in vitro* battery of tests of increasing complexity to identify complex mixtures with the potential for chronic toxicity (eg. mutation, cancer)[2]. Others have estimated the hazard associated with pollutants by identifying the chemical components of a complex mixture. This information is then used in combination with computer programs designed to estimate their biological activity (of the mixture). Athough both approaches provide valuable information concerning sample activity, they do not estimate the potential synergistic or antagonistic effects a pollutant may exhibit in the intact animal. An *in vivo* or combined *in vivo*/*in vitro* toxicological test system may best approximate this situation.

The Health Effects Research Laboratory at Research Triangle Park, NC (HERL/RTP, NC) has developed an *in vivo*/*in vitro* toxicological screen for the identification of biologically active hazardous waste mixtures. The screen involves the short term repeated exposure of rodents to waste materials followed by toxicological assessment of the whole animal, body

tissues and fluids. Emphasis is placed on identifying the type of biological activity associated with wastes in addition to the degree of activity.

The scientists responsible for the program's design and the areas of evaluation included in the study are Dr. E. Berman for general toxicology (gen tox), Drs. J. Allen, L. Claxton, J. Lewtas and T.K. Rao for genetic toxicology (gene tox), Dr. L. Reiter for neurotoxicology (neurotox), Dr. R. Smialowicz for immunotoxicology (immunotox), Dr. J. Laskey for reproductive toxicology (reprotox), Dr. N. Chernoff for teratology, and Dr. J. Creason for statistical evaluation and consultation. Dr. L. Hall is responsible for the selection of the doses for control and test compounds. The overall study design, coordination and management is under the direction of Dr. J. Lewtas and Ms. B. Andon.

The protocol, called the Toxicological Screen (Tox Screen), has been developed to provide the informational bases required for aiding regulatory judgments regarding the classification of wastes under the Resource Conservation and Recovery Act (RCRA). Any waste classified (or listed) as hazardous would be subject to stringent disposal methods as specified under Subtitle C of RCRA.

The Tox Screen has been reviewed by an external committee of scientists[3]. Their recommendations and comments have been incorporated into the Tox Screen's design. Additional review will be made during the validation of the Tox Screen's protocol in an effort to ensure the quality of the test methods developed.

This paper discusses the design and validation of the hazardous waste Toxicological Screen.

Methods

The protocol for the Toxicological Screen can be divided into two sections. The first is the treatment protocol which defines the animal exposure regimen. The second is the biological screens which includes the toxicological assessments performed with the whole animal or various body tissues and fluids (Figures 1 and 2). Only the logistics of performing the screen as a single protocol are described here. Specific protocols concerning the biological endpoints (i.e. procedural details, equipment, reagents, etc.) are beyond the scope of this paper.

The Treatment Protocol

Seventy day old Fischer 344 male rats are exposed by gavage to the waste mixture for 10 consecutive days. Five concentrations of the test material and two negative control concentrations (cage and vehicle) are utilized. The maximum concentration delivered to the animal is 1 gram/kilogram/day (g/kg/day). The remaining 4 doses are reduced logarithimically usually by factors of Log 2 or 3 (Figure 1). Exposure of the test material is adjusted according to the weight gain or loss of the animal. The animals are weighed on days 0, 3, 6 and 10 of the 10 day study. The dosing volume is calculated for each rat based on its latest weight. The neat waste material and extracts of the wastes are employed for *in vivo* exposure.

Under this protocol, 7 groups of animals are exposed to the wastes. The cage control and 2 of the 5 treated groups each have 10 animals. The vehicle control group and 3 of the 5 treated groups each have 14 animals/group, bringing the total number to 86 animals. The extra four animals/group are utilized for sister chromatid exchange analysis.

Biological Endpoints

During or at the end of the 10 day treatment the whole animal and/or body tissues and fluids are evaluated in a series of toxicological assays. These assays are listed in Figures 1 and 2. A time-course description of the order in which these assays are performed is given below and in Figure 3.

Mutagens In The Urine

The mutagenic evaluation of urine from rats gavaged with waste samples will assist in identifying metabolic and physiological events which occur in the intact organism and which can not be duplicated in vitro. Body fluid analysis will be performed in an attempt to identify the in vivo formation of mutagens[4]. Results from this study will play a critical role in determining the relative risk of genetic damage resulting from exposure to waste samples.

Immediately following the administration of the 5th consecutive dose (day 5), twenty-four-hour urine samples are collected from one animal in each treatment group. The 7 animals are randomly selected and placed in 7 nalgene metabolism cages. Each cage is suspended over dry ice to ensure the integrity of the urine samples. Immediately following collection (day 6) the raw urine is evaluated for the presence of mutagens using the Ames Test. Each urine sample is evaluated using strains TA98 and TA100 at 3 to 5 concentrations (depending on sample size) in triplicate both with and without a rat liver microsomal preparation.

In Vivo Sister Chromatid Exchange (SCE)

Sister chromatid exchange analysis has become widely used as a sensitive measure of induced chromosomal alterations[5]. In the present study, in vivo methodological approaches were used to measure SCE frequencies in treated rat bone marrow cells.

On the morning following the 10th consecutive dose, four animals designated for SCE studies from three of the five treated groups and the vehicle control group are pretreated with bromodeoxyuridine. These 16 animals are then given colchicine on day 11 followed by the harvesting of bone marrow cells for SCE analysis.

Neurotoxicology Screen

Because behavorial and neurological complaints are reported by people exposed to industrial wastes, it is necessary to employ rapid neurotoxicological tests to provide an indication of the hazard posed by specific waste samples. The neurotoxic potential of waste samples will be assessed by routine short-term behavorial tests including motor activity and acoustic startle response[6,7]. These behavorial tests are among the most rapid and reliable tests of neurotoxicity.

Twenty four hours after the 10th consecutive dose (day 11 of the study) 10 animals from each concentration group are evaluated in the neurotox screen. This involves the assessment of motor activity using the figure 8 maze and response to auditory stimuli using the acoustic startle response test.

Remaining Screens

The following determinations are performed on each animal unless

otherwise stated.

On day 12 the same 10 animals evaluated in the neutrotox screen are anesthetized, weighed, and exsanguinated through the abdominal aorta. A small portion of whole blood (0.5 ml) is placed in a test tube for leukocyte count determinations. A blood smear is also made for differential leukocyte counts. Serum is prepared from the remaining blood for liver function and other serum chemistry tests, testosterone and FSH determinations. These parameters are used to assess specific and general somatic normality. In order to determine the immunotoxic potential of wastes the thymus and spleen are removed aseptically and weighed. Spleen cells from each concentration group are utilized for mitogen response and natural killer (NK) cell assays. The mitogen assay is a good *in vitro* correlate of *in vivo* T-and B-lymphocyte function[8]. Natural killer cells are a nonspecific first line of defense against tumors, certain viruses, and certain infectious agents. The 4 hour ^{51}chromium release assay employed here for NK activity correlates very well with *in vivo* tumor model assays[9].

For an initial assessment of reproductive toxicity, the testes and epididymides are removed and weighed. The right testis from each animal is trimmed and placed in Bouin's fixative for pathological examination. The epididymal fluid from the left cavda epididymis is utilized for studies of sperm concentration, motility and abnormal spermatoza. Sperm head counts are made with the left testis. These physiological measurements are common for assessment of risk in the male rat[10].

As part of the necropsy performed on day 12, urine is collected directly from the bladder with a sterile syringe and applied to an Ames' Bili-Labstix. Each animal is then examined for gross thoracic, abdominal, and external pathology including examination of the heart, lung, liver,

kidney, and adrenal. The liver, kidney, and adrenals are weighed and organ-to-body-weight ratios are calculated to assess specific organ toxicity and stress response[11]. Except for the spleen, testes and epididymides all animals and organs are discarded at the end of experimental day 12.

Teratology Screen (Neonatal Survival)

The neonatal survival screen, which was developed at HERL/RTP, is a simple, cost-effective test[12]. Its results are highly correlated with those obtained in the standard teratology protocol. It relies upon the well-known observation that rodent pups born with birth defects are generally cannibalized by the dam, and that those which do survive show significant growth deficits. These end points -- death and weight loss during the first 3 days postpartum -- are extremely easy to measure. This screen, therefore, provides a simple way to identify agents having the potential to adversely affect the embryo/fetus.

Forty eight (24 treated; 24 controls) pregnant Fischer 344 female rats are exposed by gavage to one concentration of the waste material beginning on day 7 of gestation and ending on day 16 (10 consecutive days). The selection of the single exposure concentration for neonatal survival is made near or at the maximum tolerable dose level observed for the males in the Tox Screen. The dosing volume is calculated based on the initial weight of the female on day 6 of gestation. This dosing volume is utilized for 10 consecutive days. The change in maternal weight during the treatment period is determined by weighing the dams after the 10th dose. The animals are allowed to give birth (day 22 of gestation) and the litters are counted and weighed at birth and at 3 days

of age. The resulting litters are randomly reduced to one male and one female. At 3 weeks of age the pups are evaluated in the neurotox screen; at 6 weeks the pups are killed for the immuno tox screen. This sequence is shown in Figures 2 and 3.

SCE Analysis With Maternal Lymphocytes and Embryo Liver

In order to determine if transplacental SCE inductions occur with treatment of the dams with wastes, SCE frequencies in maternal lymphocytes and embryo liver cells are compared to control levels.

After the 8th consecutive dose maternal blood is collected from the 8 animals designated for SCE analysis (4 treated, 4 control). The blood samples are cultured for lymphocyte SCE analysis. Immediately following blood removal, the dams are administered 5-bromodeoxyuridine. The following day the animals are killed and embryo livers are assessed for SCE frequencies.

Validation of the Toxicological Screen

Before the Toxicological Screen can be utilized with complex waste samples, its ability to detect biologically active compounds and complex waste mixtures must be determined. This first goal of the validation study is being accomplished by using a series of positive and negative control compounds with the entire protocol. The positive control compounds selected for study are well characterized chronic toxins of two basic types. The first type causes a wide variety of chronic toxic effects (e.g., mutagenic, immunotoxic and teratogenic etc.). Cyclophosphamide, the first control compound evaluated in the Tox Screen, is a good example of this type of compound. The second type has a very narrow and specific activity (neurotoxic only, etc.). After a prescribed number of individual

compounds have been evaluated, additional validation will be accomplished with the use of well characterized complex waste mixtures.

Cyclophosphamide, chlordecone, acrylamide, and diethylstilbestrol (DES) are the first control compounds being evaluated using the entire Tox Screen. Exposure concentrations for each chemical are selected based on literature data for oral short-term and sub-chronic studies. For SCE analysis only three treated concentrations and one vehicle control group are evaluated. As part of the neonatal survival experiment dams are exposed to one concentration of the control compounds.

The use of control compounds will allow assessment of the 10 day screen's ability as a predictive tool for known chronic effects. The evaluation of compounds of known chronic effects in the Tox Screen may allow for a comparison of the predictability of results obtained in these short-term tests for known chronic effects. The potential identification of chronic effects with waste samples would be of great value when considering disposal of the wastes.

A second goal of the validation study is to identify which biological endpoints are most predictive of the toxic potential of the pure control compounds. Control compounds which are known to produce one or a minimum number of toxic responses but are detected in other areas provide data concerning the sensitivity of the tests. Those compounds possessing a wide variety of activity will identify tests responding in agreement with known effects. With a combination of both sets of information it may be possible to identify one or several tests from the Tox Screen which has the greatest potential for identifying biologically active test materials.

The identification of those tests which are most sensitive plays an important role in the validation study. Many compounds must be evaluated

in the Tox Screen before such assays will be statistically apparent. Presently, a minimum of 20 pure compounds have been suggested. If such assays are identified they can be combined to form a prescreen.

The selection of a prescreen is an important aspect of the validation study. Due to the large number of RCRA samples requiring evaluation, a "mini screen" which would 1) identify grossly biologically active samples and 2) help prioritize waste for evaluation in the Tox Screen would be beneficial. Those assays selected for the prescreen would be utilized with an abbreviated form of the Tox Screen's treatment protocol.

Due to cost and time restrictions imposed upon the prescreen not all assays in the Toxicological Screen are likely candidates for the prescreen. For example the clinical chemistry tests and testicular pathology are too expensive and time consuming to meet cost and time restraints. Although all assays are being evaluated as part of the Tox Screen's validation only several tests are considered likely candidates for the prescreen.

An example of the prescreen protocol with a hypothetical selection of biological endpoints is given in Figure 4. If the results of the validation study demonstrate that changes in body weight, the Ames test with wastes, the neurotox assessments of the Figure 8 maze and acoustic startle response as the most sensitive tests in identifying biologically active samples, they will be used in combination with a treatment protocol which varies slightly from the Toxicological Screen. In the prescreen, 10 animals will be exposed by gavage to one concentration of wastes (< 1g/kg) for 10 consecutive days in an effort to facilitate screening. If it is possible to select a prescreen protocol, this approach will facilitate the identification of biologically active wastes. Should any waste evaluated in the prescreen demonstrate activity or toxicity, it will not

be re-evaluated in the prescreen at a different concentration. It will become an immediate candidate for the Tox Screen.

It is important to remember that the prescreen is designed to identify grossly active samples and to prioritize waste samples for testing in the Tox Screen. The prescreen neither characterizes activity nor provides any dose-response data for waste samples. For these reasons information obtained from the prescreen can not aid in regulatory or disposal decision making processes. In addition, any sample which demonstrates no activity in the prescreen remains a candidate for the Tox Screen but at a low priority level. Failure to respond in the prescreen does not constitute a biologically "inactive" waste sample.

An integral part of the research program is to develop dose response relationships for waste samples. The prescreen tests a single concentration and does not meet this criteria. For this reason any test utilized in the prescreen will not be eliminated from the Tox Screen. Samples will be reevaluated with the selected prescreen test(s) at five concentrations in an effort to generate dose-response relationships. The only exception to this would be for a totally in vitro test utilizing the neat or extracts of the wastes (ex. Ames Test). Such test will have already generated dose-response data because it does not require samples generated in vivo.

Examination of the validation study results may indicate the prescreen concept to be totally inadequate for prioritizing wastes. Should this be the case, the prescreen concept will be eliminated. The screening of hazardous wastes would then commence with the Toxicological Screen.

Currently, the validation study is designed to include several tests from six areas of toxicology. Such a design is intended to maximize the probability of detecting biologically active wastes. It is possible

however, that one or several of the tests included may prove to be relatively uninformative, insensitive or redundant as to the type and degree of information it provides. The results of the validation study will allow assessment of each test with respect to the above points. This assessment may result in the elemination of any test(s) demonstrating little utility and merit. This approach will help assure the Tox Screen's efficiency for screening waste samples both across and within the six disciplines of toxicology.

Criteria For Test Selection

Each biological screen used in the Tox Screen will be evaluated with positive and negative control compounds. The test results will be scored in comparison to the known toxicity of control compounds. The criteria for test selection will be based on the sensitivity, specificity, and accuracy scores for each test as shown in Figure 5. These scores will be determined individually for each biological screen as judged against the positive and negative compounds selected to evaluate that seven (e.g., the Immunotox Screens will be scored against known positive and negative immunotoxins).

In order to evaluate each biological screen as a candidate for the prescreen test, the sensitivity of each test in predicting other toxicities will be determined (e.g., the Immunotox Screens will be scored against known positive and negative carcinogens, teratogens, etc.). Sensitivity will be optimized so that a minimum number of biological screens could be selected to detect any potential toxicity. Accuracy will be maximized to the extent possible, however, specificity will not be a critical consideration in selection of prescreen tests.

Evaluation of the entire Tox Screen's utility in accurately confirming the toxicity of a waste mixture and in determining the toxic endpoints effected by the waste mixture will rely more heavily on the specificity score.

The diverse dose levels employed in the entire Tox Screen coupled with the different biological screens should also permit the ranking of toxic endpoints effected as a function of dose for each waste mixture evaluated.

Discussion

The Toxicological Screen has been developed to identify potentially hazardous waste samples and process stream residuals. The experimental design of the screen was chosen to meet three goals.

The first goal was to maximize the amount and type of information obtained under a given protocol. This has been accomplished by combining the in vivo exposure with in vitro methods in 6 areas of toxicology. Using a single group of animals (except for teratology) maximizes the amount of information obtained while reducing cost. The type of activity each sample possesses is defined with evaluation in the many biological endpoints employed. Waste samples demonstrating a great degree of activity in any of the six areas of toxicology will be candidates for more sophisticated and detailed studies. The knowledge of the area of toxicity which detects the toxic potential of the complex waste at a low concentration level relative to the other areas will aid in selecting tests for future studies. Emphasis can be directed towards elucidating initial observations in a specific discipline in addition to continued investigation in all areas.

The second goal was to determine at what concentration a toxic effect can be observed by exposing the animals to five concentrations of the test material. This establishment of a dose-response relationship will aid in determining the relative risk of exposure to the waste. Where feasible a comparison between humans and animals will be made based on dose response relationships. This will result in the generation of a hazardous waste data base which will allow the comparison of the relative toxicity of various waste mixtures and will directly support risk assessment.

In 1976 Congress enacted RCRA to eliminate unsound waste management practices. This act has been hampered, however, by the lack of adequate test methods for hazardous waste identification and hence their proper disposal. Consequently, the third goal of the Tox Screen is to develop its methodology to provide adequate data for use by EPA's Office of Solid Waste in listing wastes in compliance with RCRA.

Conclusion

The development of technologies to control hazardous wastes is critical to EPA's mission to guarantee the protection of public health and the environment from the adverse effects of hazardous wastes. The search for effective technologies is complicated by the large number of hazardous waste streams resulting from numerous industrial processes. The Toxicological Screen presented here is currently being evaluated for its ability to detect a wide variety of biologically active wastes in a timely, cost-effective manner. Evaluation of wastes in six areas of toxicology will increase the likelihood of identifying toxic wastes, while providing a basis for waste regulation and risk assessment.

Acknowledgements

The authors would like to thank A. Hunter for the preparation of this manuscript.

Disclaimer

The research described in this paper has been reviewed by the Health Effects Research Laboratory, US Environmental Protection Agency, and approved for publication. Mention of trade names or commercial products does not constitute an endorsement by the Agency or recommendation for use.

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Figure Legends

Figure 1 - Toxicological Screen.

Figure 2 - Teratology portion of Toxicological Screen.

Figure 3 - Schematic overview of the Toxicological Screen.

Figure 4 - Hypothetical prescreen protocol.

Figure 5 - Definition of scores useful in the prescreen test selection.

Toxicological Screen

Treatment Protocol

- One species: rats, Fischer 344
- One sex: males
- One age group: adults (70 days)
- One route of exposure: gavage
- Dose: 5 doses
- Length of treatment: 10 consecutive days

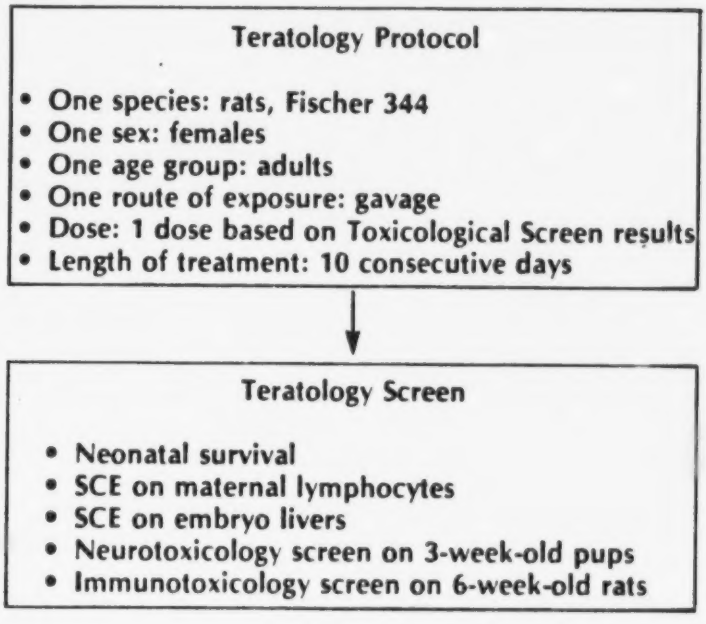
→ Teratology Protocol (based on Toxicological Screen results)

Biological Screens

General Toxicology	Mutagenesis/Carcinogenesis	Neurotoxicology	Immunotoxicology	Reproduction

• Death
• Weight change
• Organ/body weight ratios:
  ▪ Kidney
  ▪ Adrenals
  ▪ Liver
• Gross necropsy
• Clinical tests:
  ▪ Urinalysis
  ▪ Liver chemistry
  ▪ Serum chemistry
	• Bioassay of body fluids and tissues
	  ▪ Mutagens in urine
	  ▪ *In vivo* sister chromatid exchange in bone marrow
	• Direct bioassay of sample
	  ▪ Point mutation in bacteria
		• Motor activity: Figure 8 maze
		• Acoustic startle response
			• Hematology
			• Organ/body weight ratios:
			  ▪ Spleen
			  ▪ Thymus
			• Mitogen response
			• Natural killer cell activity
				• Testes weight
				• Sperm concentration
				• Sperm motility
				• Testosterone level
				• FSH levels
				• Testicular pathology

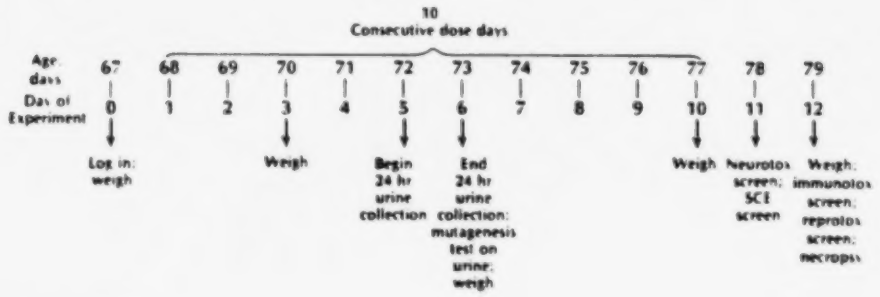
Figure 1


Teratology Protocol
• One species: rats, Fischer 344
• One sex: females
• One age group: adults
• One route of exposure: gavage
• Dose: 1 dose based on Toxicological Screen results
• Length of treatment: 10 consecutive days
Teratology Screen
• Neonatal survival
• SCE on maternal lymphocytes
• SCE on embryo livers
• Neurotoxicology screen on 3-week-old pups
• Immunotoxicology screen on 6-week-old rats

Teratology Portion of Toxicological Screen

Figure 2

Schematic Overview of the Toxicological Screen


10
Consecutive dose days
Age, days
67 68 69 70 71 72 73 74 75 76 77 78 79
Day of Experiment
0 1 2 3 4 5 6 7 8 9 10 11 12
Log in; weigh
Weigh
Begin 24 hr urine collection
End 24 hr urine collection; mutagenesis test on urine; weigh
Weigh
Neurotox screen; SCE screen
Weigh; immunotox screen; reprotox screen; necropsy

Teratology Screen

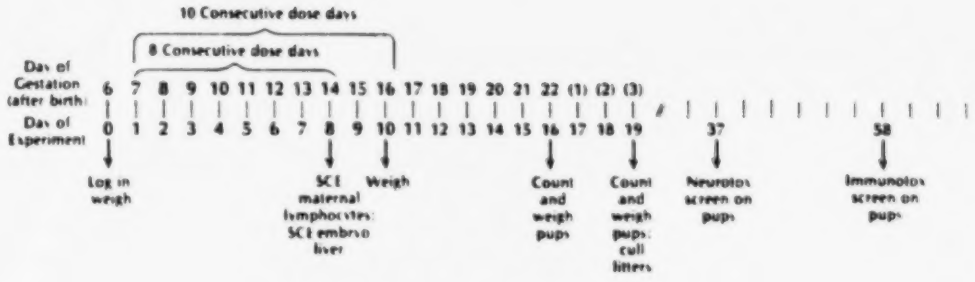

10 Consecutive dose days
8 Consecutive dose days
Day of Gestation (after birth)
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 (1) (2) (3)
Day of Experiment
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 37 58
Log in weigh
SCE maternal lymphocytes; SCE embryo liver
Weigh
Count and weigh pups
Count and weigh pups; cull litters
Neurotox screen on pups
Immunotox screen on pups

Figure 3

Hypothetical Prescreen Protocol

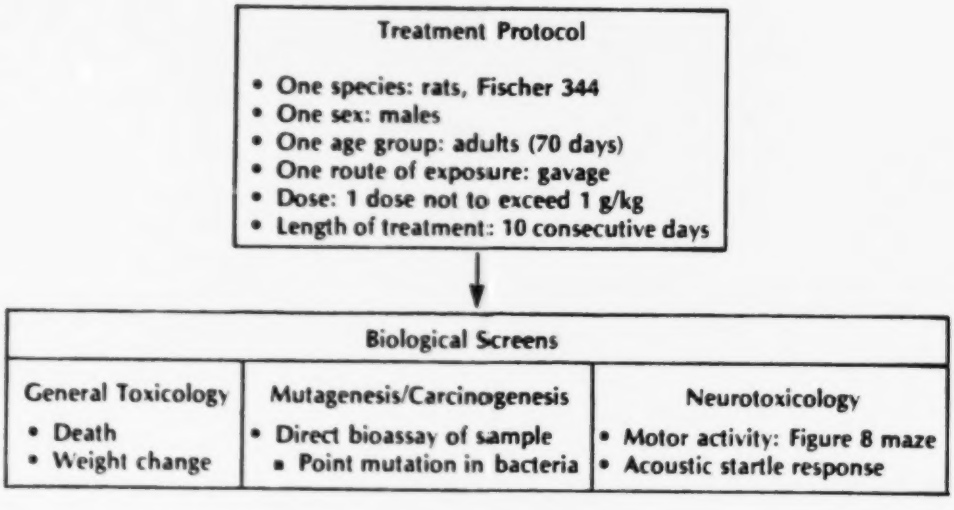

Treatment Protocol
• One species: rats, Fischer 344
• One sex: males
• One age group: adults (70 days)
• One route of exposure: gavage
• Dose: 1 dose not to exceed 1 g/kg
• Length of treatment: 10 consecutive days
Biological Screens
General Toxicology
• Death
• Weight change
Mutagenesis/Carcinogenesis
• Direct bioassay of sample
▪ Point mutation in bacteria
Neurotoxicology
• Motor activity: Figure 8 maze
• Acoustic startle response

Figure 4

Definition of Scores Useful in Prescreen Test Selection

		Control Compound +	Control Compound −	
Test Result	+	a	b	a + b
	−	c	d	c + d
		a + c	b + d	N

Several useful scores for each test may be defined as follows:

Sensitivity = a/(a + c)
Specificity = d/(b + d)
Accuracy = (a + d)/N

Figure 5